An investigation on the Interaction between Hsp90 and p53 in Breast Cancer: An In-Silico approach.

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

Master of Technology

in

Biotechnology

By

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Certificate

This is to certify that the thesis entitled "**An investigation on the Interaction between Hsp90 and p53 in Breast Cancer: An In-Silico approach.**" by **Smita Priyadarshini Pilla (210bm2322)** submitted to the National Institute of Technology, Rourkela for the Degree of Master of Technology is a record of bonafide research work, carried out by her in the Department of Biotechnology and Medical Engineering under my supervision and guidance. To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/ Institute for the award of any Degree or Diploma.

Dr. **Subhankar Paul**

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Acknowledgement

If words are reflected as symbols of appreciation and token of acknowledgement, then words play the role of thanks to exhibit the deeply embedded feeling of gratitude. I am greatly indebted to, who either through guidance, discussion or providing facilities for the thesis work, have served as a beacon light or crowned my efforts with success. With an overwhelming sense of pride and genuine obligation I express my deep sense of heartfelt gratitude and regards to my guide **Dr. Subhankar Paul**, Department of Biotechnology and Medical Engineering for giving me an opportunity to do the project work in an independent arena.

I consider it a privilege to express my gratefulness to **Dr. Bibekanand Mallick**, Department of Life Science for the valuable guidance, and suggestions during the project work.

Further I would like to express my thankfulness to **Mr. Sailendra mahanta**, **Mr. Deependra Ban, Manoranjan** and **Miss Arunima** for their constant support and advice in my project work.

It is an immense pleasure to thank my friends **Binay, Deepak, Rupam, Monika, Maneesha, Divyanshu, Harsh, Shubhanwita, Dhruva** and all others for their persistent encouragement and day –to-day support.

I sincerely thank all my faculty members for their blessings.

Finally I am grateful to my parents **Mrs. Nilima Panda**, **Mr. Bibhuti ranjan Panda** and family members for their endurance, love, wishes and support, which helped me in completion of my work. Above all, I thank **Almighty** who showered his blessings upon us.

(Smita Priyadarshini Pilla)

CONTENTS

Abstract

Breast cancer is a common malignancy and a lifetime risk among females worldwide. Interestingly, it has been observed that Hsp90 is overexpressed in many breast cancer cells. Hsp90 is a highly conserved molecular chaperone and is having ATP dependent functions that involve stress response, homeostatic control, folding, stabilization, activation, and assembly of wide range of client proteins including p53 in various biological processes. Hsp90 inhibition has been reported to be a therapeutic approach in breast cancer.

In the present investigation, Hsp90 and its Hsp complex (Hsp70, Hsp40) has been docked with wild type p53 and mutant p53 with the help of Hex 6.3 docking software. The results showed that while Hsp90 alone has high affinity to bind with wild type p53, its affinity becomes high for mutant p53 when it is associated with multi-chaperone complex. In order to inhibit Hsp90 a wide range of ligands were selected and docked by Hex 6.3 keeping ATP as control. Geldanamycin having binding energy -315.36 kcal/mol showed highest inhibition among all. The ligplot 1.3.6 analysis confirmed the involvement of four residues Asp93, Phe138, Lys58, and Thr184 at the binding interface. Due to poor solubility and cytotoxicity results, Geldanamycin was modified into ten analogues using Marvinsketch tool 5.9.1

While Analogue 2 found to be the best Hsp90 inhibitor by Hex 6.3 docking, Analogue 9 however observed to be the best when docked by Autodock 4.0 software. Further docking of this inhibitory complex with mutant p53 by Hex 6.3 elucidated the effect of inhibition. Inhibited Hsp90 showed less binding affinity towards mutant p53 depicts the conformational changes upon inhibition.

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Abbreviations

Hsps	Heat Shock Proteins
Asp	Aspartic acid
Thr	Threonine
Phe	Phenylalanine
Lys	Lysine
Gln	Glutamine
His	Histidine
Arg	Arginine

CHAPTER 1: INTRODUCTION

1.1Introduction

Normally cell growth takes place in a control and co-ordinate manner. But sometimes due to external factors cells grow uncontrollably. And this uncontrolled growth leads to formation of tumor. The tumor may be a benign one or a malignant one. Benign ones do not form cancer while malignant ones not only form cancer but also metastasize to other parts of the body that lead to death. All this happen because of disturbances in cell cycle and regulatory pathways. The reason may be genetic mutation, functional aberration in cell cycle, effect of environmental factor and age factor. There are many different types of cancer affecting different parts of body, most of which are termed according to the place of origin.

1.1.1Breast cancer

Cancer is the main cause of death worldwide, approximately 7.6 million deaths (around 13% of all deaths) in 2008, with an expected 13.1 million deaths in 2030. And breast cancer covers 22.9% of all cancers in women. In 2008, breast cancer caused 13.7% of cancer deaths in women all over the world. There is a lifetime risk of developing breast cancer. The occurrence of breast cancer in India is on the escalation and is quickly becoming the number one cancer in females. The importance of the situation is apparent after working through current data from Indian Council of Medical Research (ICMR). It is testified that one in 22 women in India is expected to suffer from breast cancer during her lifespan. **(*Ferlay, J.* *et al 2007*).**

Hanahan and Weinberg (Hanahan and Weinberg et al 2000) stated that genetic disorder allows a cell to procure six characteristics that are specific to most cancers. Those are *(*i) self-capability in cell growth signaling (ii)non-responsive to anti-growth signaling (iii) ability to evade

apoptosis (iv) persistent angiogenesis (v) tissue invasion and metastasis and (vi) indefinite replicative potential.

The genetic instability of cancer cells allow them to escape the molecular targeting of signalling pathway, which makes them insensitive to targeted therapeutics. Thus continuous attack on multiple points or nodes of a cancer cell's network of overlapping signalling pathways should be affected than the inhibition of one or a few individual signalling nodes.

The maximum oncoproteins and their web are entangled with hsp90 molecular chaperone.

1.1.2 HSP90

Heat shock protein 90 (Hsp90), a molecular chaperone that plays important role in folding, stabilizing, activating and maintaining the conformational integrity of its client proteins through its ATPase activity. Some of its client proteins are Her2, Raf-1, Akt, Cdk4, polo-1 kinase, B-Raf, mutant p53, Bcr-Abl and are connected with cell regulation and signalling. Cancer cells use the Hsp90 chaperone to protect the mutated oncoproteins from misfolding and proteasomal degradation. It is found that Hsp90 ATPase activity is up regulated nearly 100 fold in cancer cells. And the reason may be the up regulation of its function

Hsp90 is a member of superfamily (includes DNA gyrase, Histidine kinase and DNA mismatch pair) that contains an ATP binding pocket which is different from ATP binding cleft of protein kinases. The evolutionary conserved chaperone structure consists of three domains. These domains are composed of nearly 732 amino acids. It has two isomers α and β, mainly present in cytosol. The N –terminal domain is an amino terminal domain that contains a fold known as bergerat fold which is having the ATP and drug binding site. The middle domain is having a co-chaperone interacting motifs that provide docking sites for client proteins and co-chaperones which play a part in forming the active ATPase. The c-terminal domain is the carboxyl terminal

domain that is having a dimerization motif, which is a second drug binding region and site of interaction of other co-chaperones. Dimerization of Hsp90 monomer via c-terminus is crucial for chaperoning function. After dimerization in its open state bind to ATP molecule hence preceding the attachment of co-chaperones and client protein. Hence inhibiting the ATPase cycle would interfere with the chaperoning function directly. The client protein those are in mutated form would go for proteasomal degradation, if the support thread of hsp90 vanishes.

1.1.3 p53

One of the most important client proteins of Hsp90 is p53, also known as "the guardian of the genome". It is a phosphoprotein that contains 393 amino acids and four domains .In normal cells p53 expression is low and which is responsible for cell cycle, DNA repair and Apoptosis. The major regulator of p53 is Mdm2, which is responsible for triggering its degradation. But the mutant form of p53 which involve in cell malignancy is found to bind with hsp90 strongly and escape the proteasomal degradation pathway. This result in high level of p53 and further it lead to increase the level of hsp90, which interfere with the normal metabolism of the cell. Therefore hsp90 is pursued as a promising target for cancer therapy.

Small molecular hsp90 inhibitors bind to ATP binding pocket that cause the inhibition of the catalytic cycle of hsp90 in the ADP bound structure, leading to the inhibition of chaperone activity. This result in proteasomal degradation of oncoproteins, promotes cell cycle arrest and consequent apoptosis. Inhibition of hsp90 function is effective in killing cancer cells, which are resistant to therapy as kinase inhibitors. Thus Hsp90 is a promising target in cancer therapy and with the development of Hsp90-inhibitory agents in neurodegenerative diseases and pathogenic resistance (i.e., viral, fungal, bacterial) is following behind.

There are now 13 inhibitors undergoing clinical evaluation and 23 active ones for oncology trials, with many others following in late stage IND (Investigational New Drug) evaluation and pre-clinical evaluation. While the interest in inhibitors spreading fast, the knowledge on the target lagging behind. It is known that when all inhibitors in advanced stage binding to its N-terminal binding site regulatory pocket, evidence showed some important distinction in the invitro and invivo behavior. The differences are the kinetics of client protein modulation and invivo pharmacodynamics, both of which have a strong effect on the clinical efficacy and therapeutic window. In addition it is important to identify tumor and disease specific Hsp90 clients and implement in clinic as molecular markers to get a proof that the target Hsp90 is inhibited. The cost of successful anticancer drug development to approval has scaled to more than 800 million dollar. Most molecules are known to inhibit Hsp90 function. Among which Geldanamycin was the first identified Hsp90 inhibitor and as such has served a central role in the study of hsp90 biology. For these findings, Computational tools and Bioinformatics softwares are needed. In this era Insilico approach reduces half of the cost of the research work

1.1.4 Bioinformatics and Computational Biology

Now-a-days the enhancement of Bioinformatics and Cheminformatics is paving the way for easy drug designing. Computational biology and Bioinformatics have the potential to speed up drug discovery processes, reducing the costs of the processes and changing the way the drugs are designed. Rational drug design facilitates and speeds up the drug designing processes that involves various method of identifying novel compounds. One advanced method is the docking of the drug molecule or ligand or inhibitor with the target. The site where the drug binds is known to the site of action, which is responsible for the pharmaceutical effect is the target.

Docking is the method by which two molecules bind to each other in 3D space. In addition, regression based or knowledge based scoring functions can be useful to compute the free enthalpy of ligand binding. There are various tools, softwares and servers meant for docking calculations. They may be rigid, flexible, and semi flexible docking. There are different databases store macromolecular 3D structure and ligand structure, which are extracted from NMR co-ordinates used for docking and simulations. Thus computational biology or In Silico approach is developing day by day with refinement. It is becoming a promising field and with the help of this the time and cost of biological work related to drug discovery, molecular interaction is reducing.

1.2 Objective

- To investigate the binding affinity of the interaction between Hsp90 and p53.
- To identify the amino acid residues located at the binding interface.
- To design novel Geldanamycin analogues as Hsp90-inhibitors.
- To analyze the effect of Hsp90 inhibition of various novel inhibitors on the binding affinity between Hsp90 and p53.

1.3 Plan of Work

Plan of Work	3rd Semester		4th Semester	
	Mid Semester	End Semester	Mid Semester	End Semester
Literature Review				
Exploration of Hsp90 and p53 interaction By Hex 6.3 and Analysis.				
Docking calculation on Hsp90-Ligand binding by Hex6.3				
Docking calculation on Hsp90-Geldanamycin Analogues binding by Autodock 4.0 and Ligplot analysis.				
Manuscript Writing				

CHAPTER 2 : LITERATURE REVIEW

2. Literature review

2.1 Heat shock proteins(also known as molecular chaperones)

When a cell experiences environmental stress, either its cycle stops, or slows down its original functions, such as transportation, DNA, RNA and protein synthesis. However, a set of proteins, called stress proteins, which are mainly expressed under these adverse conditions of stress response in rise in the outside temperature, called heat shock proteins.

In **1962 Feruccio Ritossa** discovered the heat shock response, who observed an amplification of special sections of Drosophila melanogaster chromosomes (heat shock puffs) when heat treatment was given to the flies.

Most of the heat shock proteins are molecular chaperones

In **1996 Hartl** defined chaperone as "proteins that bind to and stabilize an otherwise unstable form of other protein and, by exact binding and release, enable its correct fate *in vivo*: be it folding, oligomer assembly, transportation to a certain subcellular compartment, or removal by degradation"

In **1996 Multhoff and Hightower et al** discovered the expression of heat shock proteins on cancer cell surface.

2.2 Heat Shock Protein 90KDa (HSP90)

Hsp90 is a cytosolic protein, nearly present 1-2% in the cytosol. Its concentration varies depending on cells. (Lai et al 1984, Nollen and Morimoto 2002, Ghaemmaghami et al 2003). It is over expressed in cancer cells and estimated to be 2.8% in colon cancer cells. (Neckers 2002, Whitesell and Lindquist 2005, Pick et al 2007, Wang et al 2010, Kubota et al 2010).

2.3 The structure of Hsp90

The eukaryotic structure of Hsp90 having 40% similarity with its prokaryotic form (Farrelly and Finkelstein 1984, Bardwell and Craig 1987) and is a dimeric phosphoprotein (Spence and Georgopoulos, 1989).Hsp90 contains two chaperone-sites, one on its N-terminal domain, and other one on the C-terminal domain. There are also other binding sites for calmodulin, peptidyl prolyl isomerases and other co-chaperones. Hsp90 forms dimers.ATP binds to its N-terminal domain, and changes its conformation. (Minami et al 1994, Prodromou et al 1997, Stebbins et al 1997, Csermely 1998, Maruya et al 1999)

Hsp90 contains an ATP binding pocket in Its N-terminal domain, where Geldanamycin binds. (Stebbins et al., 1997, Obermann et al 1998, Reviewed by Joachimiak 1997) and yeast Hsp82 possess an adenine nucleotide binding site (Prodromou et al 1997, revised view in Prodromou et al., 2000). Comparing the structures of N-terminal domain of human Hsp90 without ligand and with ATP / AMPPCP was given by Li et al 2012. Human Hsp90 alpha consists of approximately 732 amino acids, three domains (N-terminal-terminal and middle) and a charge linker. (Huai Q. et al 2005, Harris S. F. et al 2004, Dutta R. et al 2000).Co-chaperones and client proteins association regulate the ATPase activity of Hsp90 (Whitesell L. et al 2005).

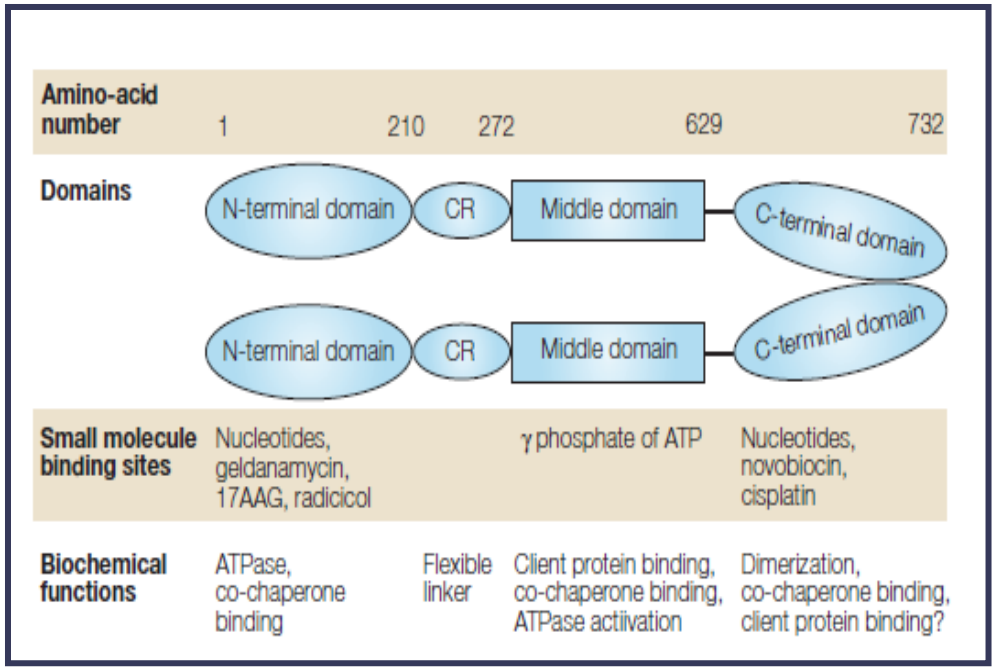

Figure 1: Structure of Hsp90 (Adapted from Ref: *Whitesell L.* *et al 2005*)

2.4 The Functions of Hsp90 in various biological processes.

Hsp90 can efficiently bind to the target proteins and is crucial for their folding, maturation and maintaining in the folding competent. (Hartl 1996, Buchner 1999, Csermely *et al* 1998, Miyata and Yahara 1992, Pratt and Toft 1997, Uma et al 1997.Members of Hsp90 binds to various peptides in vivo and in vitro .(Menoret et el 1999) Hsp90 involvement in signalling processes, poses threat to cellular function.(by Blum et al 2000). Extracellular Hsp90 interacted with the receptor CD91 (Basu et al 2001. Cheng et al 2008). Role in disassembly of transcriptional complexes: Hsp90 and p23 are recruited to chromatin-bound glucocorticoid receptor; promoter-bound p23.Hsp90 inhibit GR, TR, NFκB, an AP-1 (Freeman and Yamamoto 2002)

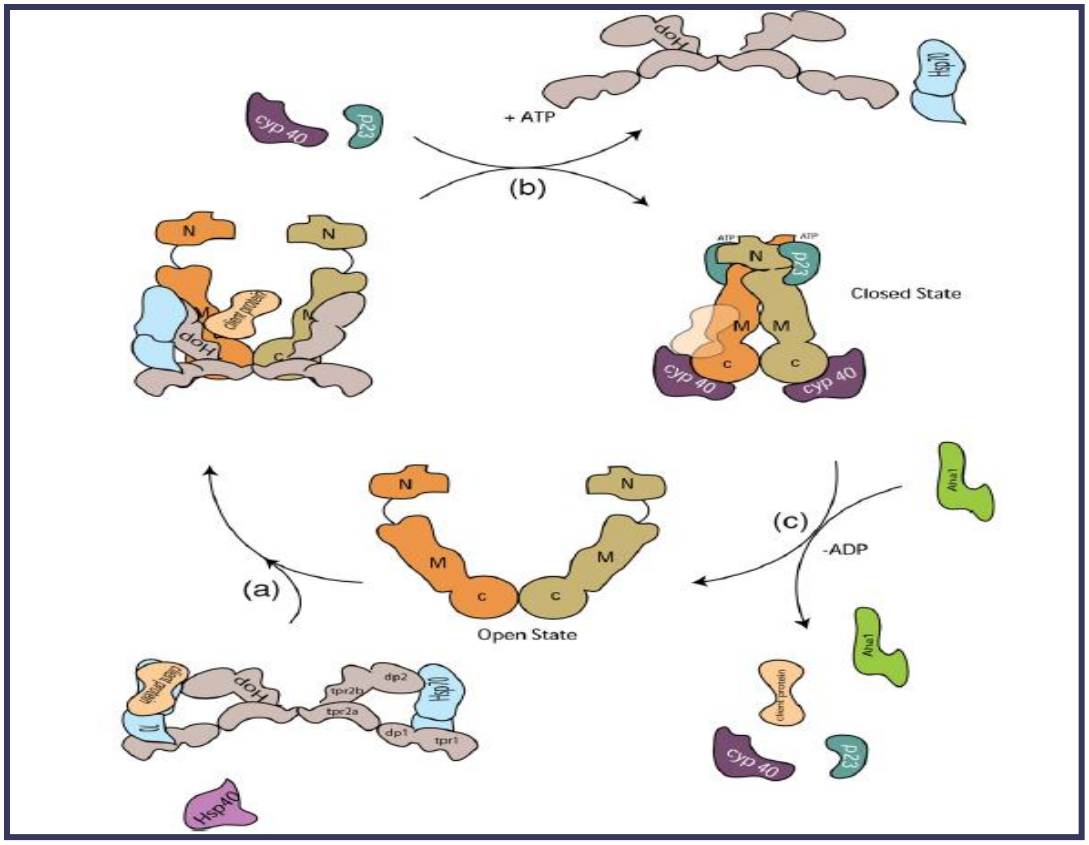

Figure 2: The Model of the conformational cycle of Hsp90 (Adapted from Ref: *Grossmann J. G. et al 2008*)

2.5 Overexpression of Hsp90 in Breast cancer cells.

Hsp90 interacts with a number of proteins in breast carcinogenesis. Hsp90 equivalents include estrogen receptors, p53 protein, hypoxia-induced transcription factor HIF-1alpha, protein kinase Akt, Raf-1 MAP kinase and a number of receptor tyrosine kinases, such as erbB2 (Beliakoff J 2004).Elevation of Hsp90 expression seems to be a trait of breast cancer and may be one of the coping mechanisms that cancer cells exhibit under stress (Conroy SE et al 1996, Yano et al 1996). It is a promising target for breast cancer therapy (Whitesell L. 2004) High Hsp90

expression level is detected in invasive breast carcinomas (Zagouri F 2008) and is associated with reduced survival in breast cancer (E Pick 2007)

2.6 Studies on the interaction between Hsp90 and its client p53.

It has been seen that Hsp90 stabilizes p53 mutants (Blagosklonny et al 1995- 1996, Sepehrnia et al 1996,Nagata et al 1999, Whitesell et al 1998) and associated Mdm2, by blocking the E3 ubiquitin ligase activity of the Mdm2 that can degrade mutant p53.(Peng et al 2001, Li et al 2011).

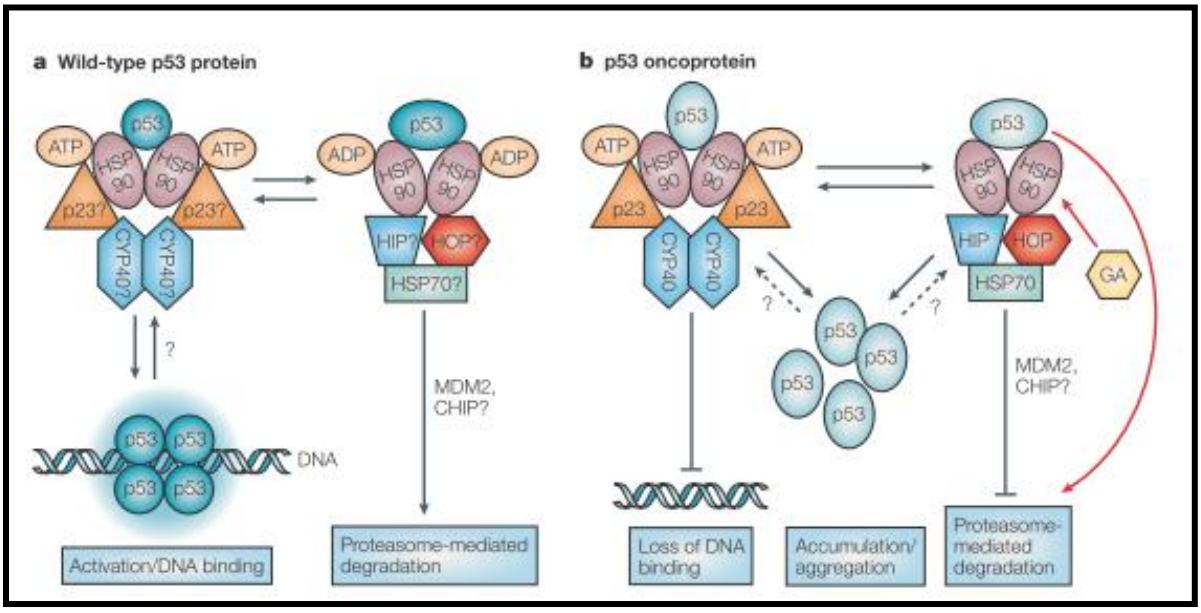

Figure 3: Depiction of chaperone interactions that control the function of wild type p53 and mutant p53 (Adapted from Ref: *Cook P. H. et al 1998)*

In in vitro analysis, hsp90 binds to wild-type p53 in a BAG-1 sensitive fashion (King et al 2001). By NMR, p53 core domain is bound to Hsp90, is primarily unstructured (Rüdiger et al 2002). Hsp90 Promotes p53 tetramer stabilization and promotes activity of Mdm2 (Burch et al 2004). Cancer cells with mutant p53 are more sensitive to Hsp90 and HDAC inhibitors than cells with wild type p53 (Li et al 2011).Hsp90 binds and stabilizes native p53 conformation (Müller et al

2004, Walerych et al 2004, Hagn et al 2011).It is required for the protection of proteins under heat stress and for refolding in collaboration with Hop-Hsp70 (Walerych et al 2009). Release of partially unfolded wild-type p53 from Hsp90 is required for promoter binding and dependent on ATP binding, but not on ATP hydrolysis (Walerych et al 2010). Interaction of Hsp90 with p53 has seen in several Hsp90 domains, including the outside of middle domain (Hagn et al 2011).

The p53 DBD undergoes loosened molten globule-like state on interaction with the Hsp90 N and M domains (Park et al 2011). Others find no evidence of conformational changes, p53 being bound in a native state based on shape and charge (Hagn et al 2011).

2.7 Hsp 90 Inhibition

There are various natural and synthetic molecules that have been declared as promising in cancer therapy via disrupting the complex of ATP-HSP90-client proteins by targeting hsp90 n-terminal ATP binding pocket. Geldanamycin was the first inhibitor that led the way for other inhibitors to represent Hsp90 a therapeutic target for cancer therapy. (Schulte et al 1995) Hsp90 binding drugs: deoxyspergualin (Nadeau et al 1994) and Geldanamycin and relatives (Whitesell et al 1994).

Geldanamycin A and Herbimycin A induce degradation of Raf, receptor tyrosine kinases (Sepp-Lorenzino et al 1995, Schulte et al 1997), CFTR (Loo et al 1998) through proteasome, and transfected nNOS (Bender et al 1999). Cancer cells with mutant p53 are more sensitive to Hsp90 inhibitors than cells with wild type p53.Oxime derivative of Radicicol with better pharmacology (Soga et al 1999).).Coumarin antibiotics such as Novobiocin bind Hsp90 and reduce levels of Hsp90 clients (Marcu et al 2000). Geldanamycin A derivative WX514 .17aag ,17dmag expresses much improve results.(Clarke et al 2000,Xu et al 2001, Elizabeth R. Glaze 2005) Geldanamycin stimulate association of CHIP with client (erbB2) and facilitates degradation (Xu et al 2002,

Zhou et al 2003) and targets tumor cells because of a 100-fold higher affinity of their Hsp90 complexes (Kamal et al 2003. A review on Hsp90 inhibition to elucidate a new strategy for protein kinases has been done. (Sreedhar et al 2004).

A new class of Hsp90 inhibitors by structure based drug designing has been discovered. (Paul Brough 2005). Other novel Hsp90 inhibitors discovered the natural triterpenoids celastrol and gedunin, by chemical genomics (Hieronymus et al 2006, Lamb et al 2006). Celastrol interrupts Hsp90 interaction with Cdc37 and function without hindering ATP binding (Zhang et al 2008, Zhang et al 2009).The involvement of computational biology and the docking studies on anticancer drugs has been much helped (Alex Mathew 2009).A review on all Hsp90 inhibitors up to date given by HUIFANG HAO 2010, Detailed thermodynamic analysis of drug binding to human and yeast Hsp90 (Zubrienė et al 2010). GA/17AAG target VDAC resulting in membrane depolarization of mitochondria and increased intracellular Ca2+ (Xie et al 2011).3D structure elucidation and macromolecular interactions on hsp90 help in establish drug designing (T. Madej 2011).

Chapter 3: MATERIALS AND METHODS

3. Materials and Methods

3.1 Tools and bioinformatics softwares used

3.1.1 Pubchem (pubchem.ncbi.nlm.nih.gov)

3.1.2 NCBI

3.1.3 Swissprot

3.1.4 Expassy tools

3.1.5 Molinspiron Server

3.1.6 PHYRE 2 server (**P**rotein **H**omology/analog**Y** **R**ecognition **E**ngine)

3.1.7 Chimera 1.6.1

3.1.8 PDB(pdb.org)

3.1.9 Blast

3.1.10 Clustalw

3.1.11 Open babel software 2.3.1

3.1.12 PRODRG 2.5 server(beta)

3.1.13 Marvin sketch 5.9.1

3.1.14 Rasmol

3.1.15 Hex 6.3

3.1.16 Ligand Scout 3.2

3.1.17 Molegro virtual Docker 5

3.1.18 Autodock 4.0

3.1.19 Ligplot + 1.3.6 and Dimplot

3.2 Protocol followed

3.2.1 Interaction between Hsp90 and p53

Retrieval of amino acid sequences of Hsp90 protein from NCBI

↓

3D structure of Hsp90 Protein was modeled and obtained by PHYRE server.

↓

3-D structure of p53, Hsp70, and Hsp40 is retrieved from PDB (Protein Data bank).

↓

Energy minimization of all 3D structure of proteins had done by Chimera.

↓

Docking of individual protein and protein complex respectively had done by Hex 6.3.

↓

Retrieval of the residues involve at the binding site by Dimplot analysis.

3.2.2 Inhibition of Hsp90

Retrieval of 3D structure Hsp90 domain was from PDB (1YET).

↓

A collection of small ligand molecule was from Pubchem database.

↓

OpenBabel 2.3.1 converted all the ligand structures from .sd file format to .pdb format.

↓

Modification of ligand molecules was by Marvin sketch 5.9.1.

↓

Energy minimization of ligands was by Prodrg server.

↓

Docking of hsp90 with all ligands was done by Hex 6.3 and Autodock 4.0.

↓

Analysis of the binding energy obtained from docking.

↓

Proposed Pharmacophore model was by LIGAND SCOUT 3.2.

↓

Retrieval of the residues involve in ligand protein interaction by Ligplot 1.3.6 analysis.

3.2.3 Effect of inhibition on binding affinity of hsp90 and p53.

Retrieval of the protein-ligand complex was done after Hex docking.

↓

Docking of the complex with mutant p53 protein structure was by HEX 6.3.

↓

Analysis of the total energy values changes before and after inhibition.

3.3 Methodology

3.3.1 Retrieval of amino acid sequences of Hsp90 protein from NCBI

NCBI stands for National Centre for Biotechnological Information. It is established as a division of National Library of Medicines at National Institutes of Health. The NCBI responsible for creating automated systems of knowledge about molecular biology, biochemistry, and genetics, providing the use of such databases and software by the research and medical community; collect biotechnology information both nationally and internationally; and execution research on advanced methods of computer-based information processing for examining the structure and function of biologically important molecules. The URL for this database is http://www.ncbi.nlm.nih.gov.

i. The above mentioned URL was browsed.

ii. In search option, protein was mentioned and hsp90 human was typed.

iii. There were many results of the search but the accession number AAI21063 was selected and the sequences were retrieved in fasta format.

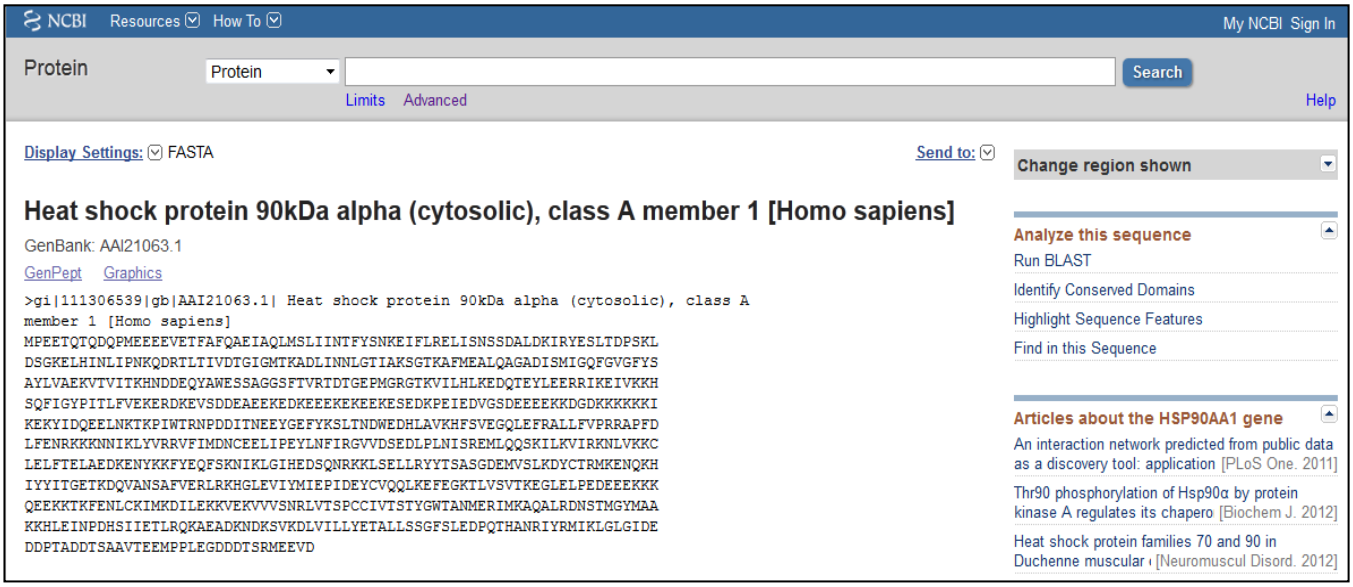

Figure 4: Retrieval of amino acids from NCBI

3.3.2 Retrieval of 3D structure of Hsp90 Protein modeled by PHYRE server.

PHYRE is an automatic fold recognition server for calculating the structure and function of the protein sequence that got submitted in the server. It is used for academic users only. It uses the principle and technique of Homology Modeling and relies on Hidden Markov Models.

i. The Fasta format of amino acid sequences was pasted and Phyre 2 search was clicked.

ii. After 5 hours the results were sent to the user given email address.

iii. The modeled structure was retrieved from the link sent by the server.

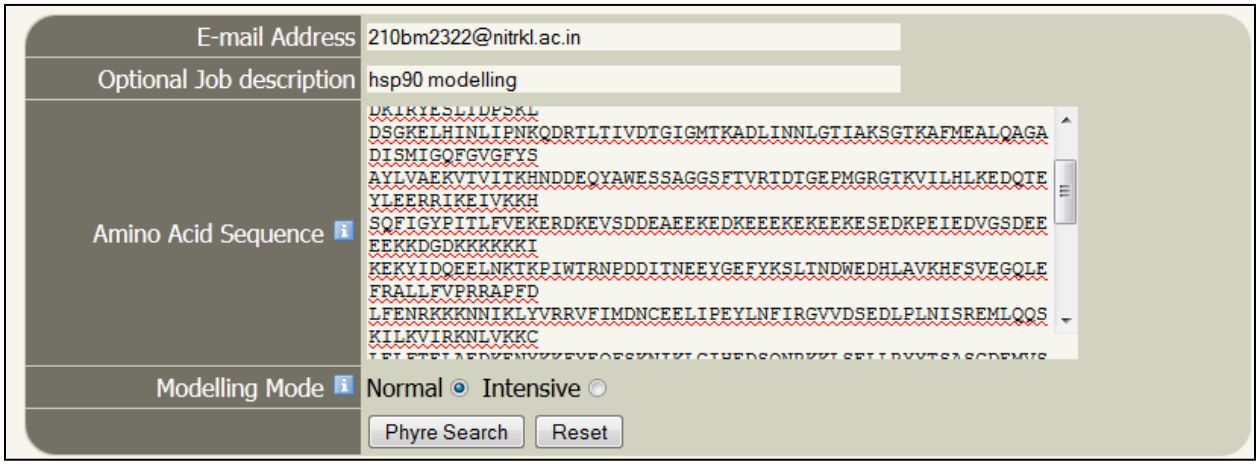

Figure 5: Submission of amino acids in PHYRE server

3.3.3 Retrieval of 3-D protein structure of p53, Hsp70, Hsp40 From PDB (Protein Data bank).

The PDB (Protein Data Bank) is the universal store of Structural data of Biological macromolecules, founded in Brookhaven National Laboratories (BNL) in 1971.It provides Structural information of the macromolecules assessed by X-ray crystallographic, NMR Methods. This is very much important as the understanding of shape will lead to the way

how it functions. As biological macromolecule like protein is having a structure to function relationship. Hence an accurate knowledge of structure is needed to know the varying functions. This server is free of use.one can easily download the structure in pdb file format or fasta format.

i. The URL www.pdb.org was browsed.

ii. In the search option Individual protein name was typed.

iii. After selecting the definite PDB ID, the structure was downloaded and saved in .pdb format.

iv. Human wild type p53 having PDB ID- 2OCJ, mutant p53 -2QVQ,Hsp90 N-terminal domain -1YET,Hsp90 middle domain -1HK7,Hsp40-2QLD,Hsp70-3ATU were retrieved from protein data bank (PDB).

3.3.4 Energy minimization of all 3D structure of proteins by Chimera 1.6.1.

CHIMERA 1.6.1 is an extensible programme for visualization and analysis of molecular structure and related data including density maps, supramolecular associations, sequence alignments, docking results, routes and conformational ensembles. One of the best features is the structural editing job. It can minimize the energy of molecules providing them high stability.

i. Chimera window was opened.

ii. From the option file, the 3D structure of protein was retrieved.

iii. The total residues were selected.

iv. From the tool option, by the structure editing option, minimized structure option was clicked.

v. The minimized structure was saved in .pdb format.

3.3.5 Collection of small ligand molecules from Pubchem.

Pubchem is a database of chemical structures of small organic molecules and contain information of their biological activity, origin and related literatures. It is executed and updated by NCBI and is freely available. Millions of compound structures and data seta can be freely downloaded in .sd format or chemical (CID) format

i. Pubchem page was retrieved by browsing pubchem.ncbi.nlm.nih.gov.

ii. In search bar Individual inhibitors name was typed & entered

iii. All the available ligands of therapeutic target database were retrieved in .sd file format.

iv. The ligands retrieved were Geldanamycin,17dmag,17aag,radicicol,snx-5422,puc3,curcumin,Herbimycin,witheferin,ATP,novobiocin,Taxol.

3.3.6 Conversion of all the structures from .sd file format to pdb by OpenBabel software 2.3.1.

OpenBabel 2.3.1 is a chemical toolkit designed to interpret the various language of chemical data. It allows searching, converting, and analyzing chemical data. It supports Cheminformatics, molecular modeling, and bioinformatics. It covert chemical data from one file format to another.

i. OpenBabel 2.3.1 window was opened.

ii. The input format was selected as .sd and the output format as .pdb.

iii. From the input folder option the .sd files were browsed.

iv. The output files were generated and saved in the selected folder in .pdb format.

3.3.7 Modification of ligand molecules by Marvin sketch and properties studied by Molinspiron server.

Marvinsketch is a tool for drawing chemical structures, adding or deleting functional group or atoms, queries and reactions. Assigning stereochemistry, charge, valence, radicals and isotopes to each atom can be done and moreover single, double, triple bonds and aromatic forms can also be created.

Molinspiration provides a wide range of Cheminformatics tools supporting molecule manipulation and processing, SMILES and SD file conversion, normalization of molecules, creation of tautomers, molecule disintegration, calculation of several molecular properties needed in QSAR, molecular modeling and drug design, high quality molecule representation, molecular database tools supporting substructure and similarity searches, also support fragment-based virtual screening, bioactivity prediction and data visualization.

i. Marvinsketch window was opened.
ii. The ligand .pdb format was retrieved.
iii. Addition, deletion of functional group changes were made keeping in mind to increase solubility.
iv. The new molecules were saved in .pdb format.
v. The Molinspiron server was opened and ligand .pdb file was uploaded to check the bioactivities.

3.3.8 Energy minimization of above ligands by Prodrg server.

PRODRG takes description of small molecules as pdb/MDL molfile and generate variety of topologies that may use for docking and simulations. It works mainly to stabilize the ligand molecule by minimizing its energy.

i. Prodrg beta server was opened.

ii. The pdb format of ligand was pasted in the space provided & run Prodrg.

iii. The energy minimized pdb file generated by the server was downloaded and saved for future use.

3.3.9 Docking by Hex 6.3

Hex 6.3 is an Interactive Molecular graphics program developed by Dave Ritchie for estimating docking calculations and displaying docking modes of pairs of protein and ligand molecules. HEX is used as the docking tool which calculate intermolecular "energies" by adding up all intermolecular interactions (e.g. van der Waals, electrostatic) that occur between a ligand and protein target

i. Hex manual window was opened.

ii. From the file, both receptor and ligand separately were opened from the path location defined.

iii. By the option control, docking was selected and activated.

iv. Lastly the binding energy (ΔE) produced by docking action was saved carefully.

v. The docking complex was saved from the file option in the .pdb format for future analysis.

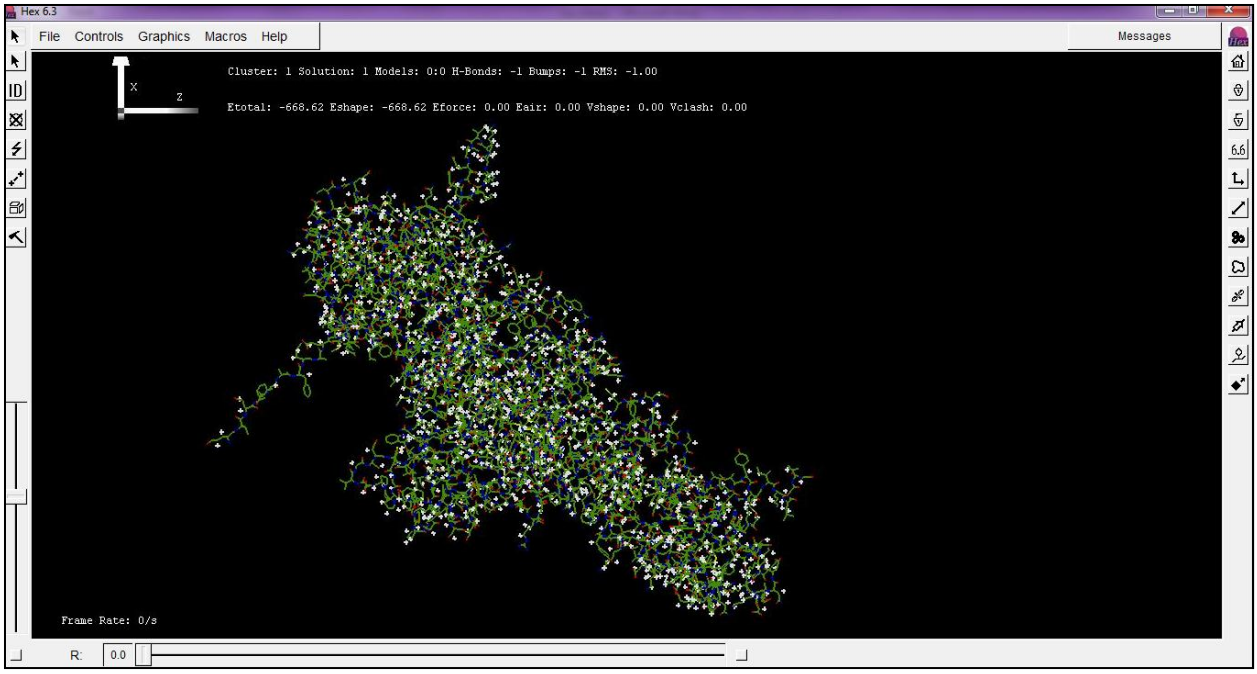

Figure 6: Docking of Hsp90 with mutant p53 by Hex 6.3

3.3.10 Docking by Autodock 4.0

Autodock 4.0 is a set of automated docking tools. It is projected to predict how small molecules or drug candidates normally bind to a receptor of known 3D structure. Autodock include 2 main programs: (I) Autodock executes the docking of the ligand to a set of grids describing the target protein and (ii) Autogrid pre-calculates these grids. The grid defined is to locate where the ligand can bind.

i. Before operating Autodock on window 7, first the startup directory path was defined. File > preferences > modify defaults >start up directory.

ii. The receptor molecule and the ligand molecules were saved in the folder location defined in directory.

iii. Autodock window was opened.

iv. The receptor file was uploaded. File>read molecule>folder> receptor.pdb> open.

v. The receptor or the protein molecule was modified. Edit>hydrogens>add.

vi. The ligand molecule was uploaded. Ligand>input>open>lig.pdb.

vii. The torsion was set. Ligand>torsion>set no of torsion>"any number">dismiss

viii. The ligand was saved in .pdbqt format. Ligand>save>lig.pdbqt>save

ix. The grid was defined. Grid>macromolecule>open>receptor.pdb>initializing ok>protein.pdbqt>save,gridbox>"set coordinates">file>close saving current>output >out.gpf>save

x. The Autogrid was run.

xi. The parameters for docking were set. Docking > macromolecule >protein.pdbqt >open > ligand > lig.pdbqt >open >accept > search parameters > genetic algorithm > 100 >ok > docking parameters > accept > output > Lamarckian parameters > dock.dpf >save.

xii. The Autodock was run for 5 to 6 hours different for different ligands.

xiii. The result was analyzed. Analyze>dockings>open>dockyyy file, macromolecule >open, conformations>play, clustering>show.

xiv. The energy values and the Ki was calculated and noted down.

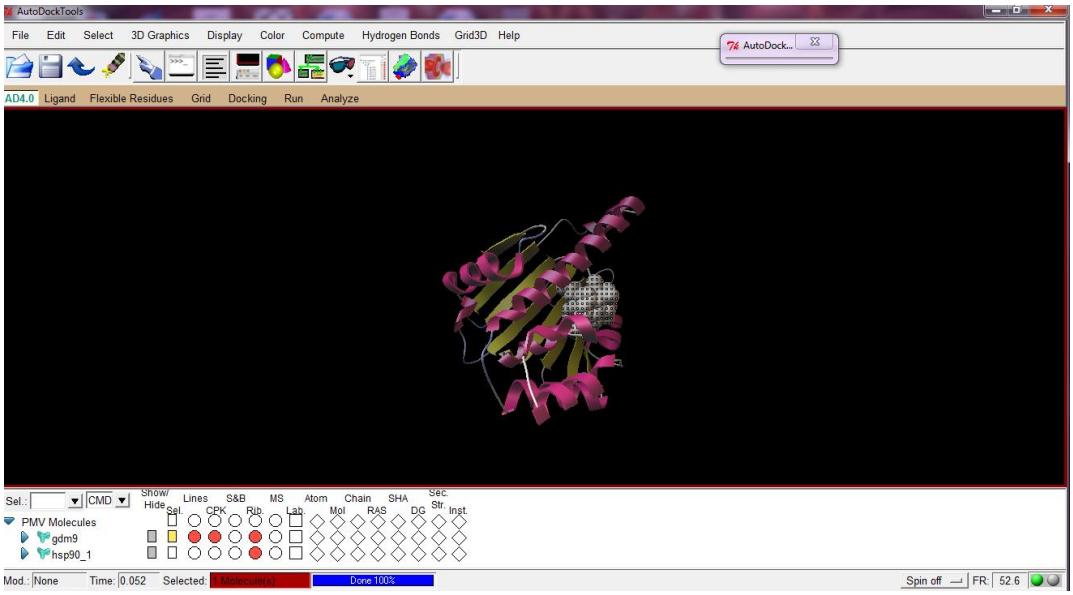

Figure 7: Docking of Geldanamycin Analogue9 with Hsp90 by Autodock tool0 by

3.3.11 Ligplot/ Dimplot analyses to interpret the residues and bonds involved in binding at the binding site.

LIGPLOT is a user friendly programme use to generate protein-ligand interactions for a given pdb file encrypting the docking. It shows the interactions facilitated by Hydrogen bonds and Hydrophobic interfaces.

i. Ligplot window was opened.

ii. The protein ligand complex.pdb file was opened.

iii. For protein –ligand interaction, ligplot analysis was done and for protein-protein interaction Dimplot analysis was done.

iv. The ligand was selected and the plot window was obtained.

v. For Dimplot protein chain was selected and the result window was opened.

vi. The plot was saved.

The ten analogoues of Geldanamycin that are created by Marvin sketch are presented below ..

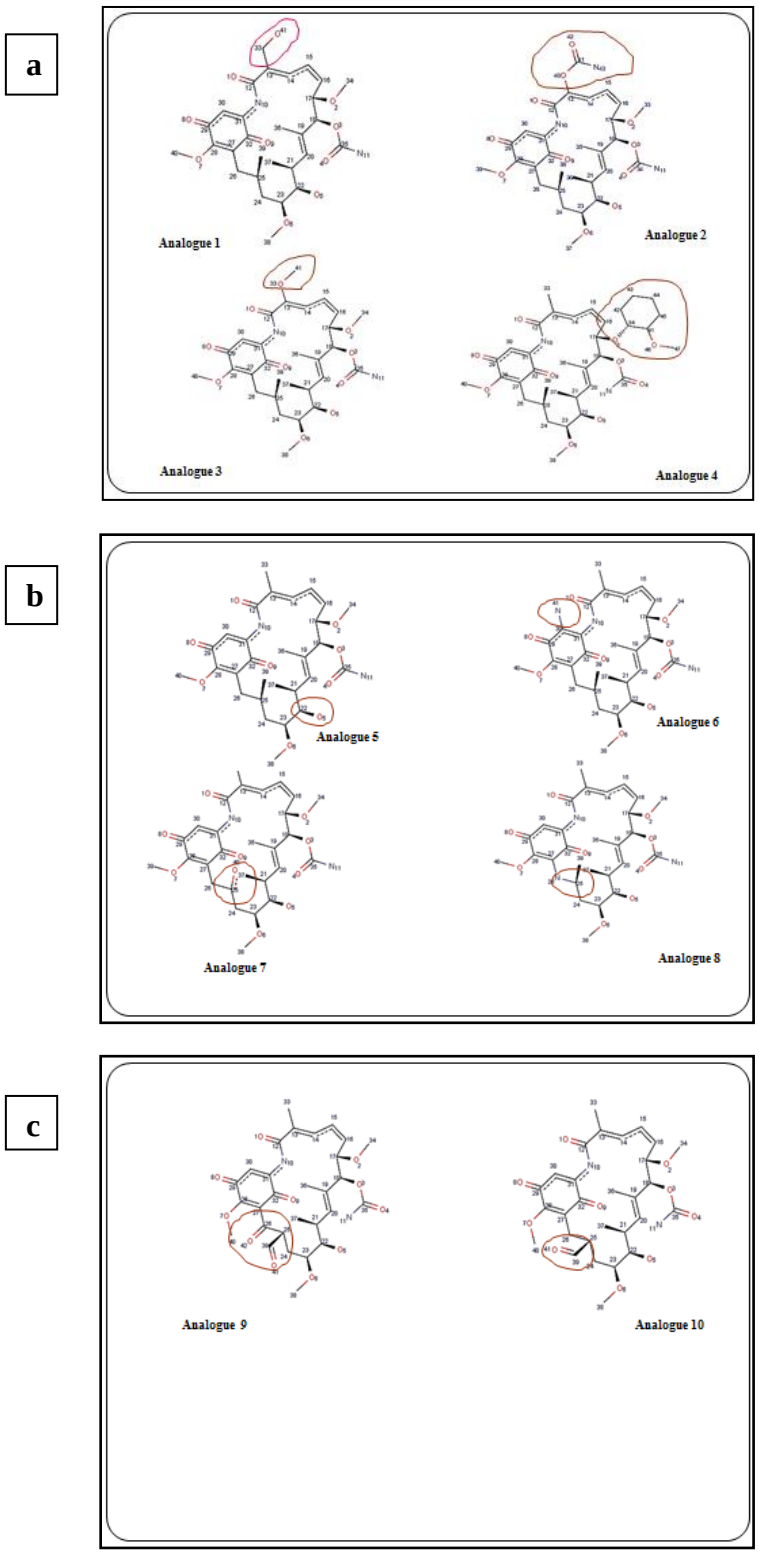

Figure 8. (a)(b)(c) Represent all the ten analogues of Geldanamycin designed by Marvin sketch 5.9.1

Chapter 4:
Results
And
Discussion

4 Results and Discussion

4.1 Interaction between Hsp90 and p53

The Hsp90 structure was modeled, aligned, and compiled in one full length 3D structure. This structure contains all the three domain:N-terminal,middle and C-terminal domain. The spiral look in the structures are α- helices and the arrow ones are β-sheets.

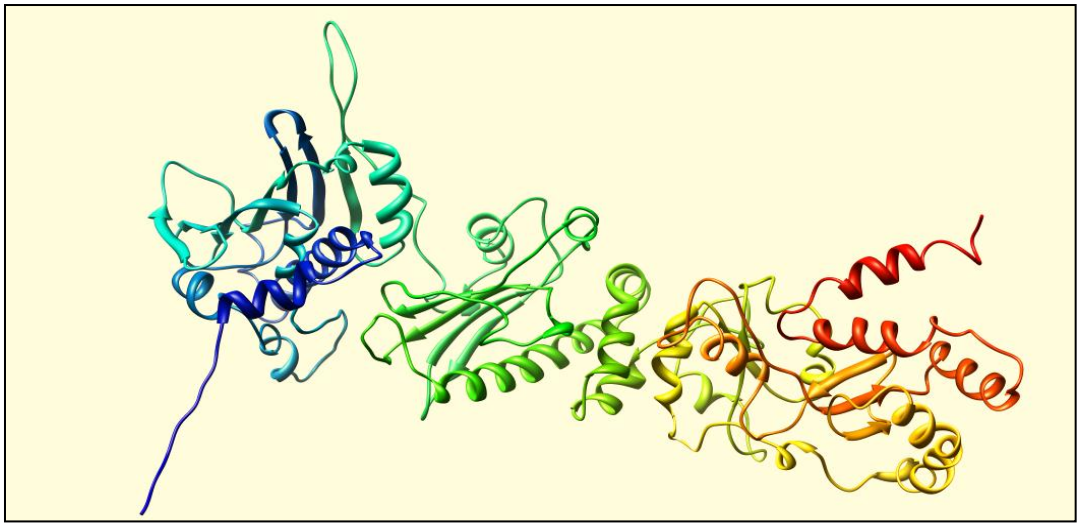

Figure 9: Modeled 3D structure of Hsp90 α by PHYRE 2 server

4.1.1 HEX 6.3 Docking Results (protein - protein)

The whole docking procedure has been done sequentially. First the Hsp90,Hsp70,and Hsp40 docked with wild type p53 and mutant p53 respectively. Then the complex of Hsp90-Hsp40,Hsp90-Hsp70,Hsp40-Hsp70,Hsp90-Hsp40-Hsp70 docked with wild type p53 and Mutant Type p53 separately. First the Hsp90 N-terminal domain was used, then followed the middle domain and the whole hsp90 full length. The docking results are summarized in Table 1.The findings of the results are solely based on the docking energy value and the interaction at the binding sites. The more negative the value, the more stable the complex is and more binding affinity. According to the energy

funnel theory less energy depicts highly stable conformation. Hence more energy would be needed to break the complex that means high dissociation energy.

Complex	Wild type p53	Mutant p53	Docking Energy
Hsp90	+	-	**- 1170.86**
	-	+	- 630.94
Hsp40	+	-	- 842.79
	-	+	- 695.12
Hsp70	+	-	- 968.13
	-	+	- 760.47
Hsp90 + Hsp40	+	-	- 736.82
	-	+	- 708.89
Hsp90 +Hsp70	+	-	- 593.33
	-	+	- 720.32
Hsp40 +Hsp70	+	-	- 497.14
	-	+	- 801.13
Hsp90+Hsp70+Hsp40	+	-	- 643.65
	-	+	**- 834.01**

Table1: Hsp90 N-terminal domain docking calculation by Hex 6.3 online server

The interpretation of the results of Table 1 is summarized below.

The docking energy of Hsp90- wild type p53 complex is -1170.86 kcal/mol and that of Hsp90 and mutant p53 complex is -630.94kcal/mol .This infer the high binding affinity of Hsp90 towards wild type p53 than mutant type p53.The Hsp90-wild type p53 complex is more stable.

The docking of Hsp40 individually or in complex form with wild type p53 came to be more stable.

The Hsp90-Hsp40-Hsp70 complex docked with wild type p53 showed energy value -643.65 kcal/mol and with mutant p53 showed -834.01 kcal/mol. Hence in complex form Hsps form stable complex with mutant p53 than wild type p53.

This interpreted that the chaperone complex activity of Hsps may involve in cancer

. Complex	Wild type p53	Mutant p53	Docking Energy
Hsp90 middle domain	+	-	- 541.06
	-	+	-686.62
Hsp90 full length	+	-	- 63.10
	-	+	-668,62

Table 2:Hsp90 other domain docking calculations.

Hsp90 other domain showed in Table 2, forming more stable complex with mutant p53 than the wild type p53.Hence there remained a strong association of Hsp90 with mutant p53.Hence proved the literature view of expression and involvement of Hsp90 in cancer. The results also proved the interaction between the Hsp90 and its client Protein p53.

4.1.2 **Results of Dimplot Analysis (for protein-protein interactions)**

The dotted line shows the hydrogen bonding ,distance between the residues, the maroon color depict the residues of Hsp90 and the pink color depict the p53 residues(both Polar and non-polar).

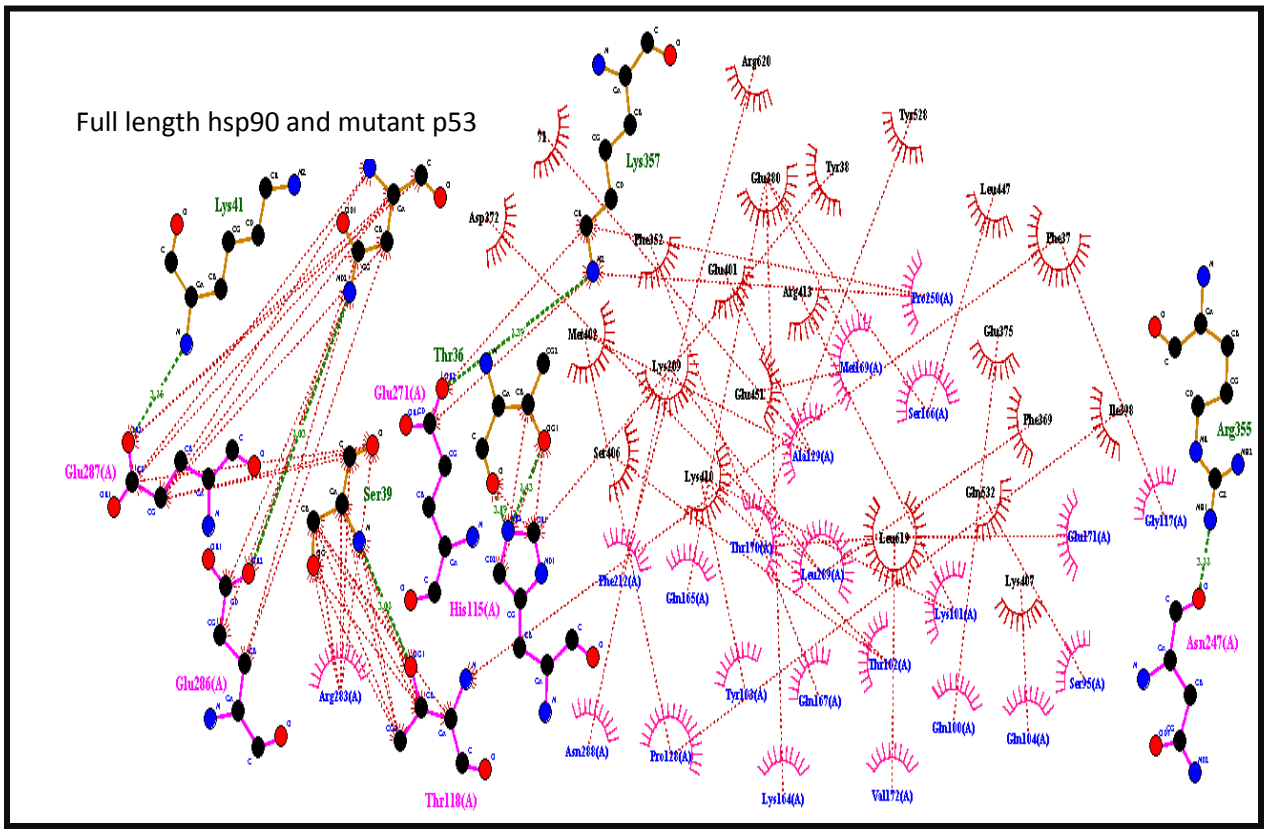

Figure 10: The Dimplot plot analysis of Full length Hsp90 and mutant p53.

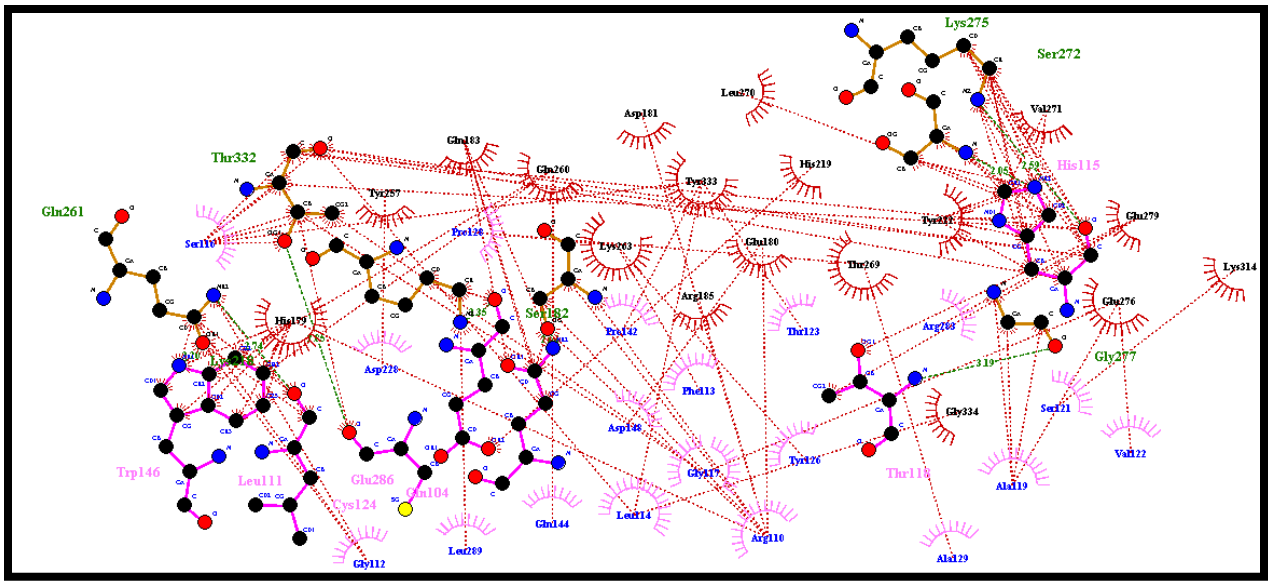

Figure 11: The Dimplot analysis of Hsp90 chain A interacting with mutant p53 chain A.

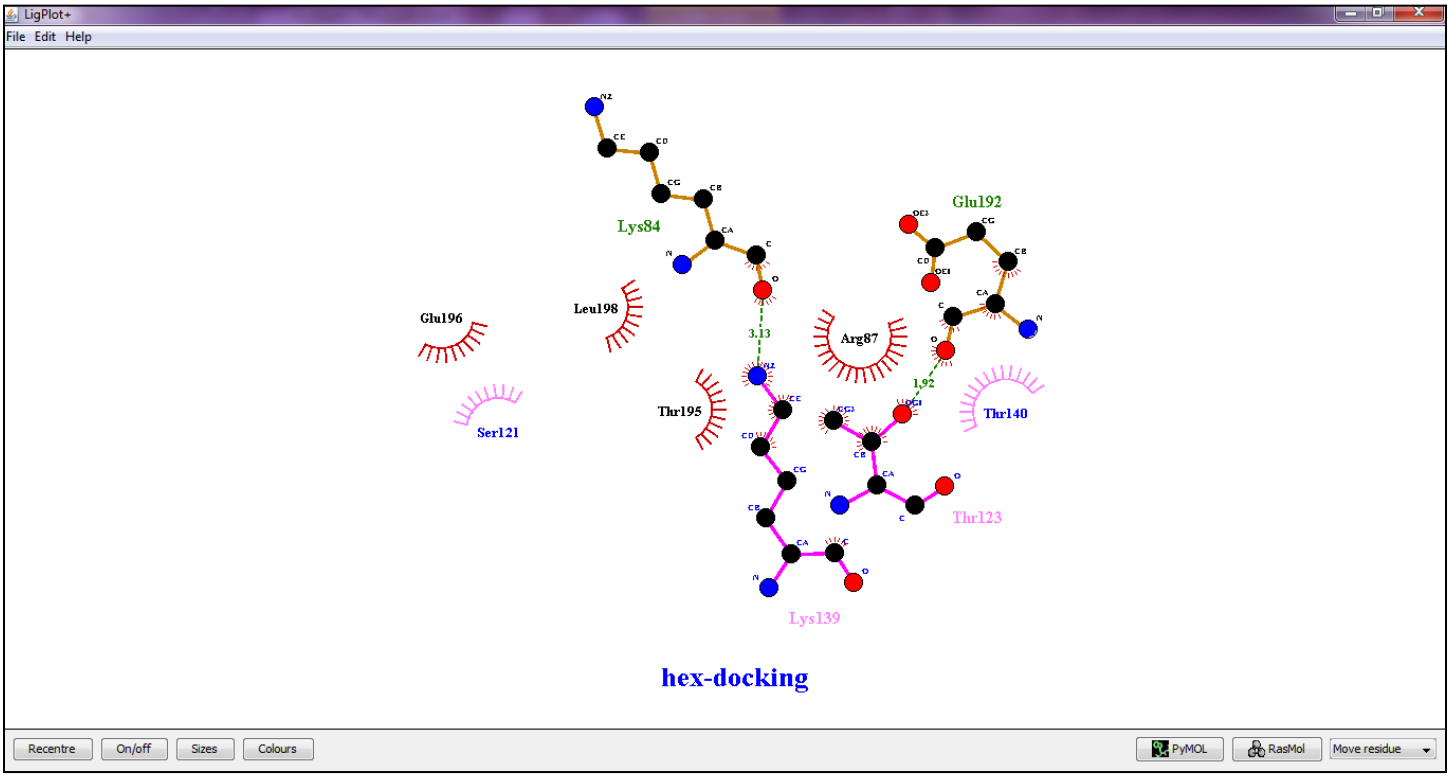

Figure 12: The Dimplot analysis of Hsp90 interacted with wild type p53

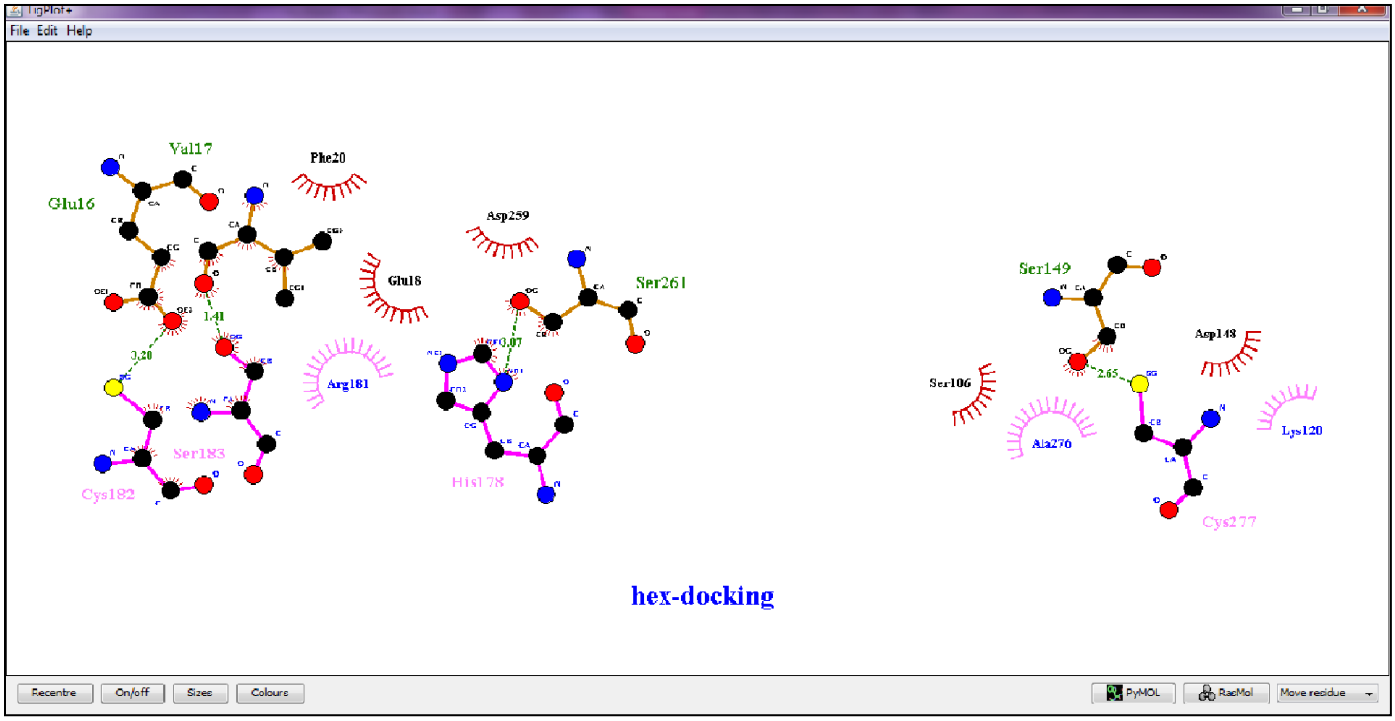

Figure 13: The Dimplot analysis of Hsp90 interacted with wild type p53. (chain A-chain B)

The results of Dimplot analysis are summarized below.

The hydrophobic residues of Hsp90 involved in the interaction with wild type p53 are Phe20,Glu18,Asp259,Ser 106,Leu198,Glu 196,Thr 195,Asp87 and the hydrophobic residues of wild type p53 are Arg 183,Ala 270,Lys120,Thr 140,Ser121.

The residues involved in hydrogen bonding are put down in Table 3.

Hsp90 residues	Wild type p53	Distance measured in Angstrom
Glu 16	Cys 182	3.20
Val17	Ser 183	1.41
Lys 84	Lys 139	3.13
Glu192	Thr 123	1.92

Table 3: The residues involved in binding of Hsp90-Wildtype p53 generated and the distance measured by Dimplot

The hydrophobic residues of Hsp90 that interacted with Mutant p53 are Arg 620,Tyr 38,Tyr 528,ser 406,Lys209,Glu 451,tyr 528,Arg 413,Lys 263,Glu180,Thr 269,Tyr 222 and the residues of p53 involved are Asp222,Gly112,Leu 289,Gln144,Tyr 126,Ala 119,Val 122.Thr 182 etc.

The residues involved in Hydrogen bonding are depicted in Table 4.

Hsp90 residues	Mutant p53	Distance measured in Angstrom
Lys 41	Glu 287	3.36
Asn 40	Glu 286	3.03
Ser39	Thr118	3.00
Thr 36	His 115	3.19,1.45
Lys 357	Glu 271	1.39
Arg 355	Asn 247	3.11
Gln 261	Leu 111	2.74
Gln 261	Trp 146	2.20
Thr 332	Cys 124	1.85
Ser 182	Gln 104	1.86
Gly 277	Thr 118	3.19
Lys 218	Glu 286	2.35
Lys 275	His 115	2.05
Ser 272	His 115	2.59

Table 4: The residues involved in binding of Hsp90-Mutant p53 generated and the distance measured by Dimplot

The above findings proved the interaction of Hsp90 and p53 (both wild and mutant), the residues involved at the binding interface and the distance measured.

4.2 Inhibition of Hsp90

The ATPase fold or the Bergerat fold on the Hsp90 N-terminal Domain has been targeted for inhibitory studies. From the Literature view point ten ligand molecules selected, keeping ATP molecule as control. As previously stated the lower the energy Value, the stronger the binding association would be. All the selected ligands were docked with Hsp90 to compete ATP molecule and the results are shown in Table5.

Ligand Molecules	**E-Value**
ATP	-262.78
Herbimycin	**-313.71**
Radicicol	-231.21
Withaferin	-276.85
Curcumin	-269.08
PU3	-257.70
Snx-5422	-293.94
Geldanamycin	**-315.36**
17AAG	-308.96
17DMAG	**-311.70**

Table 5: The docking energy calculations targeting Hsp90 by Hex 6.3

The docking of ligands was carefully observed and their interaction and orientations were also monitored. Table 5. result showed that Geldanamycin having highest binding affinity (-315.36),second to that Herbimycin also scored high (-313.71) and third one the Geldanamycin derivative 17DMAG scored good (-311.70).All the ligands except PU3 and Radicicol showed high binding affinity than the ATP molecule ,which was kept as control to row down the analysis. Hence these ligands having high affinity than ATP can be used as Hsp90 inhibitors. As the ligands are more competent than ATP molecule, that's why when they will enter the cytosol, they may replace ATP and would bind the catalytic site of Hsp90 which will further interrupt its chaperoning function.

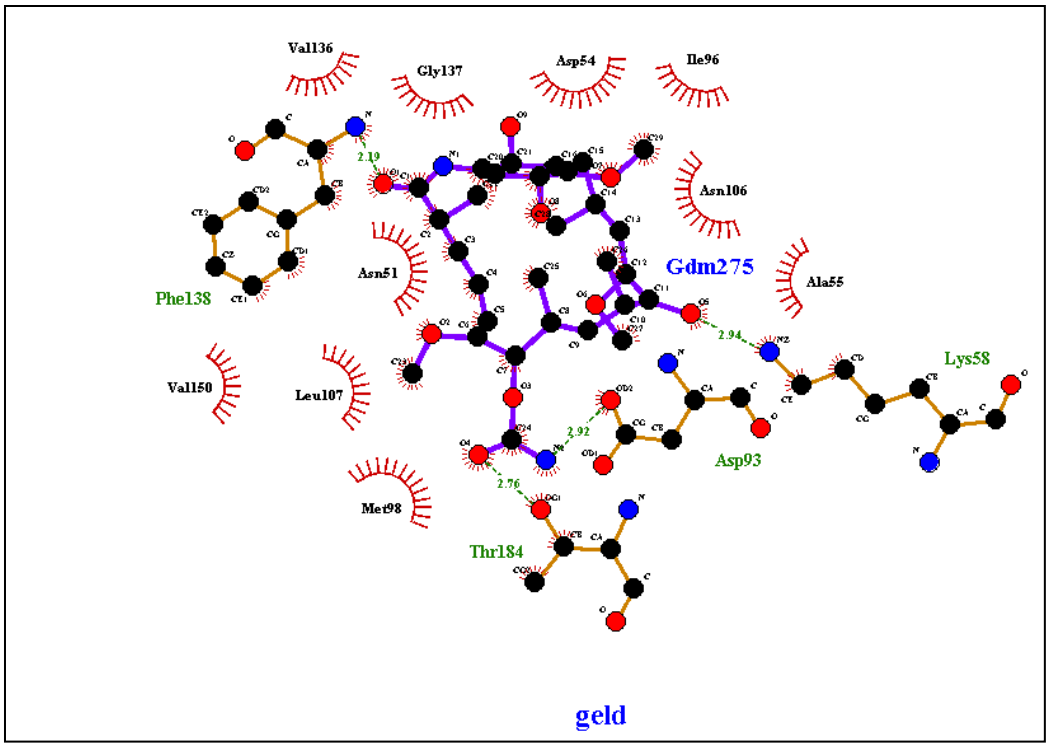

Figure 14: Showed the residues of Hsp90 interacted with Geldanamycin at the binding interface by Ligplot

The residues of Hsp90 involved in docking to Geldanamycin at the binding interface are shown in figure 6.The residues primarily involved in hydrogen bonding are Phe138,Asp 93,Thr 184,Lys

58 and hydrophobic ones are Val136,Gly137,Asp54,Ile96,Asn106,Ala 58,Met98,Leu 107 and Val180.The residues that formed hydrogen bonding are confirmed to be present at or on the binding cavity, Hence these residues would consider to be as specific ones which would involve in any further ligand binding as they are on the binding interface. Thus the four fate determining residues for other ATPase fold binders are **Phe 138, Thr 184, Lys58** and **Asp 93.**

Now, the result showed Geldanamycin is a potent inhibitor that got matched with the literature studies. According to the research works on Geldanamycin stated that it couldn't enter into the clinical trials because of its poor solubility and toxicity. The reason may be the solubility. As we know Animal cells are more aqueous in nature, hence water soluble substances easily soluble in cytosol and water insoluble form precipitates. And any extra thing in the cytosol leads to cell toxicity. This is why all the research works are going on to modify Geldanamycin structure to increase its solubility. This thought inspired us to work on the modification of Geldanamycin structure. With the help of Marvinsketch, ten analogues were made keeping in mind that it will increase solubility. Hence the functional groups that enhance solubility are –COOH,-OH,-NH, and the functional groups enhance electronegativity that will help in hydrogen bonding. Hence at different position the changes were made randomly with the functional group and somewhat hit and trial method. All the structures were tested for their bioactivity and properties on Molinspiron server.

The docking calculations were made by Hex 6.3 and tabulated in Table 6.The results showed the binding affinities of the Geldanamycin analogues .According to Hex docking Analogue 2 scored high i.e. -286.20,it mean it has high binding affinity than the others. Other analogues scored high referring to the control ATP molecule. The analogues showed good binding affinity than ATP molecule. Figure 6 showed the binding cavity of Hsp90, where Analogue 2 was bound. Figure 7

depict the map of Analogue 2 interaction on Hsp90 binding cavity. The residues involved in the interactions are Asn51, Phe138, Asp93, Ala55, Met98, Thr184, Ile 96, Leu107 and Lys112 of Hsp90 at the binding interface. The residues matched with the control that have chosen before in figure6 are Asp93, Thr184 and Phe138.Hence it is confirmed that Geldanamycin analogue 2 bind to the active site.

Geldanamycin Analogues	**E-Value**
Analogue 1	-266.17
Analogue 2	**-286.20**
Analogue 3	-270.16
Analogue 4	-267.51
Analogue 5	-270.26
Analogue 6	**-276.07**
Analogue 7	**-275.75**
Analogue 8	-270.26
Analogue9	-265.27
Analogue 10	**-277.57**

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Table 6: The docking energy calculations of Geldanamycin Analogues by HEX 6.3

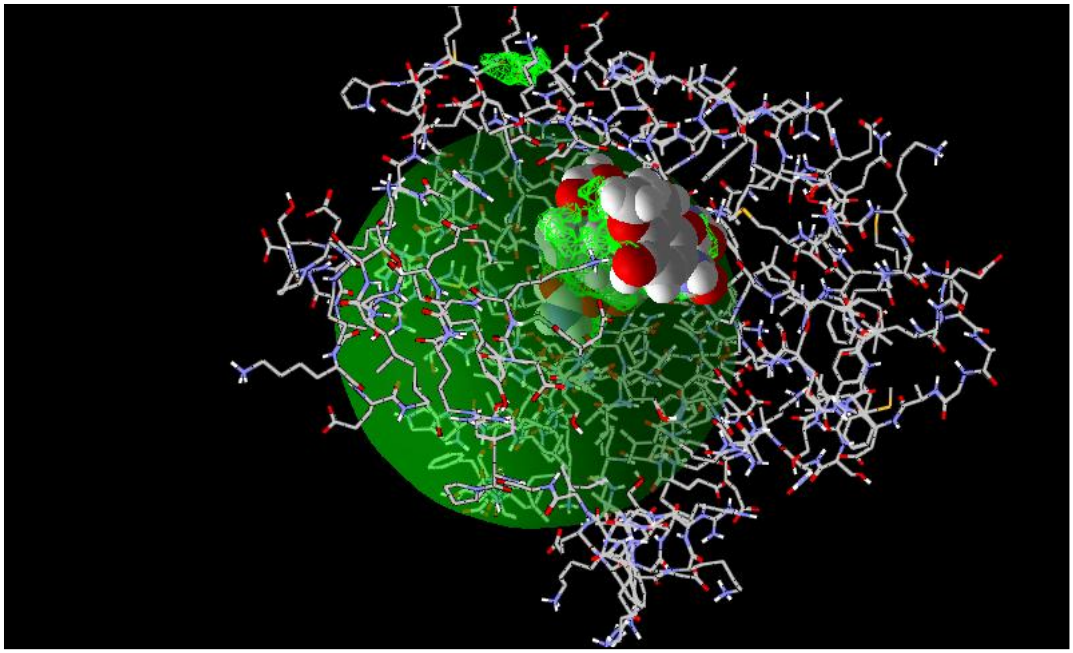

Figure 15: Geldanamycin Analogue 2 bind to the binding cavity of Hsp90 by Molegro Virtual Docker

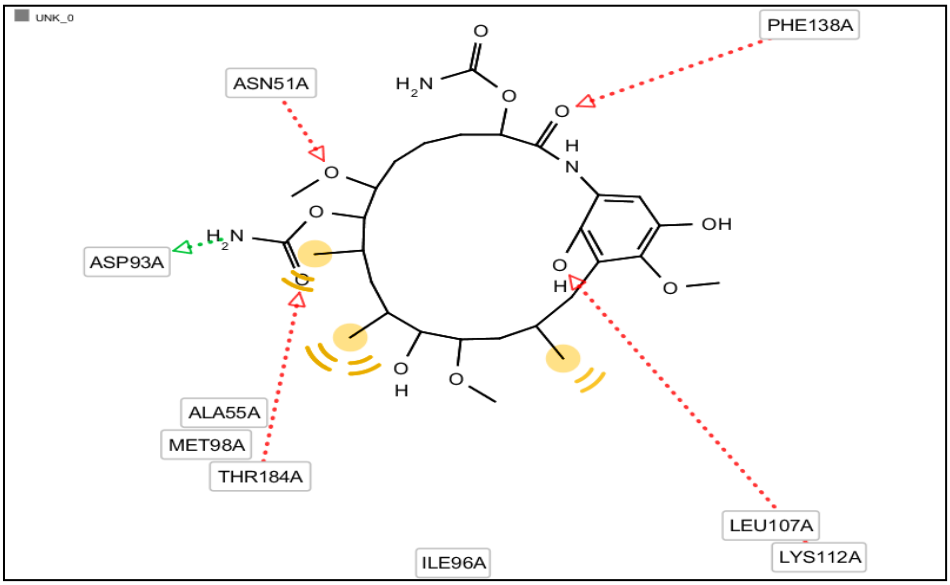

Figure 16: Analogue 2 –Hsp90 interaction map by Ligand scout

Geldanamycin and its Analogues	E-Value Kcal/mol	Ki-Value
Geldanamycin	-9.56	97.80nm
Analogue 1	**-9.63**	**86.99nm**
Analogue 2	-7.75	2.08µm
Analogue 3	-9.20	180.94nm
Analogue 4	-8.15	1.07 µm
Analogue 5	**-9.60**	**91.88nm**
Analogue 6	-9.49	110.65nm
Analogue 7	-9.44	120.26nm
Analogue 8	-9.50	108.56nm
Analogue 9	**-9.64**	**85.75nm**
Analogue 10	-9.03	239.99nm

Table 7: The docking calculations of Geldanamycin and its analogues were by **Autodock 4.0** version.

All the analogues modified were docked with Hsp90 by Autodock 4.0 version tool. Autodock is a promising tool in drug designing and docking and the work is done manually. All forms of docking, rigid, flexible and semi flexible docking can be done .Hence all the analogues were docked for 10, 50 and 100 conformations respectively. First the grid was made on whole domain to check where it is binding other than active site, and then the grid was restricted around the active site and docked. The Autodock 4.0 results varied from Hex 6.3 results. Autodock 4.0 analysis showed three analogues that outscored original Geldanamycin. Analogue 5 (-9.60 kcal/mol), Analogue 1 (-9.63 kcal/mol), Analogue 9 (-9.64) scored high that means they have high binding affinity than the original Geldanamycin.

On the basis of Ki calculation all the three mentioned ligands scored better results. Ki is the inhibition constant. It is the concentration required to produce half maximum inhibition. Hence if Ki is low, then, cytotoxicity would be low. Considering all the above facts Analogue 9 came out to be the best one.

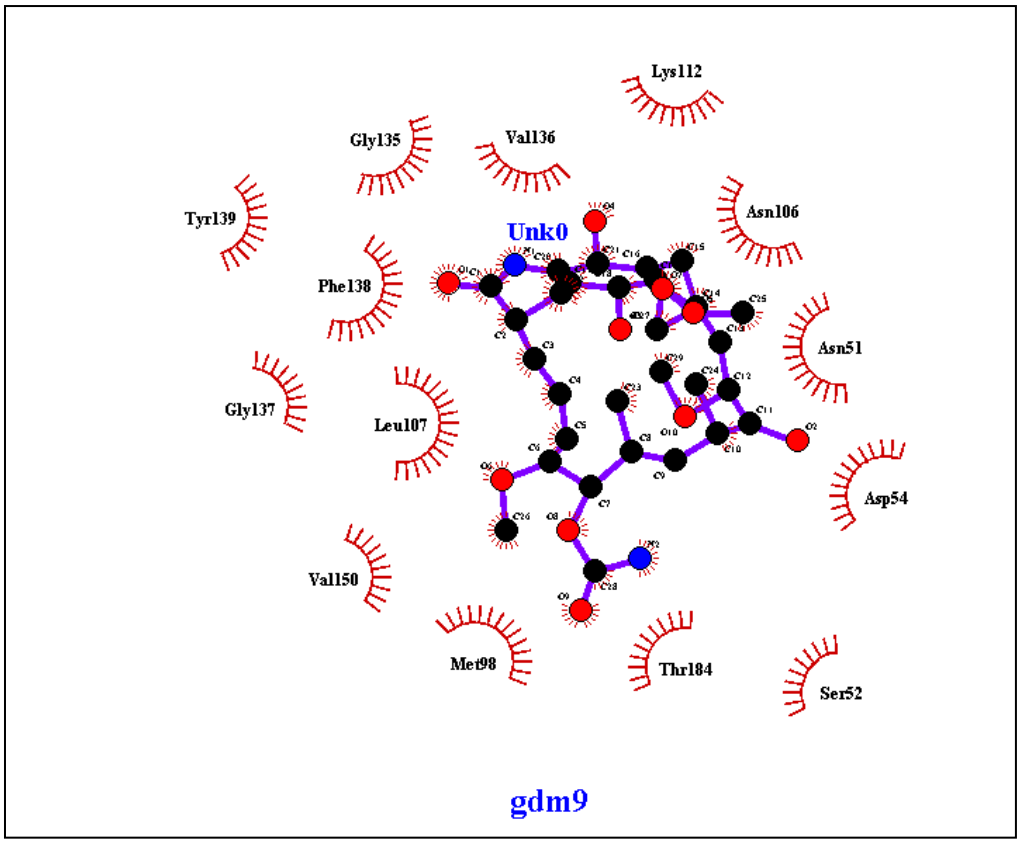

Figure 17: the hydrophobic interaction of Geldanamycin Analogue 9 at the binding cavity of Hsp90 by Ligplot analysis

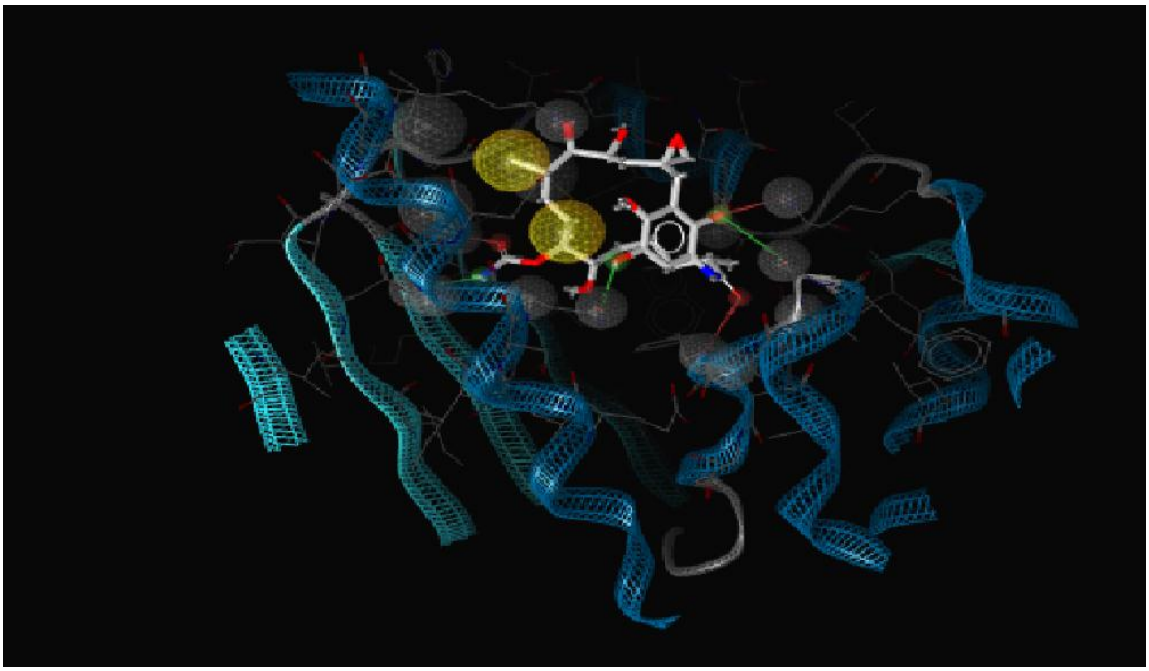

Figure 18: Pharmacophore modeling by Ligand scout

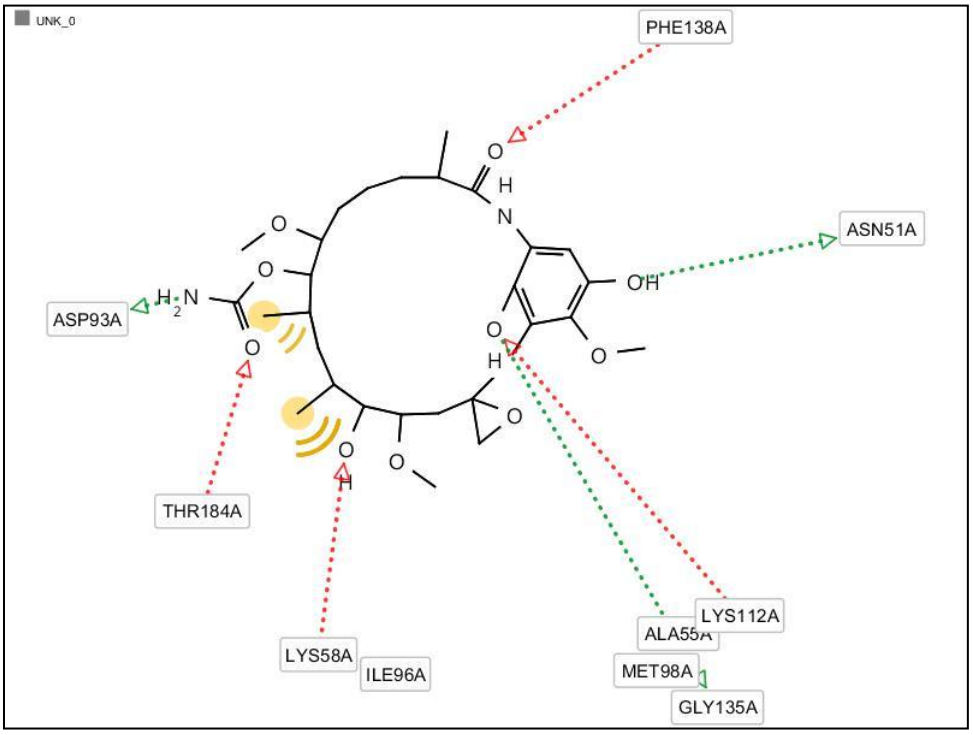

Figure 19: Ligand scout analysis of Geldanamycin Analogue9.

As the Analogue 9 showed good results, we followed to fulfill the demand of controls that have been set primarily. Figure 9 depicted the Pharmacophore modeling to enhance the binding affinity. The results of ligplot in Figure 8 and ligand scout in figure 10 added confirmation. It has been shown that the residues that involved in interaction with Analogue 9 include Asp93, Lys58, Thr184 and Phe138.Hence analogue 9 bind to the catalytic site of Hsp90 and having good affinity than the original making it a potential ligand for future uses.

4.3 Effect of inhibition on binding affinity of hsp90 and p53.

Previously the findings showed good affinity between Hsp90 and mutant p53 which worked in a chaperoning complex. For other individual domain docking with Mutant p53 established that Hsp90 is the culprit to protect its mutant form. Hence Again the Inhibition studies have undergone and some suitable candidate are found along with the Literature‘s best ones. Now To add some more spices to the study, the full length structure of Hsp90 comprising all the domains has been taken. Here Mutant p53 docked with the full length Hsp90 without any inhibitors and with specific inhibitors to analyze the consequences. All these studies were based on docking energy calculation by hex only.

Complex	Mutant p53	E-Value
Hsp90	**+**	**-668.62**
Hsp90 +17DMAG	**+**	**-619.82**
Hsp90 +Analogue 5	**+**	**-568.79**
Hsp90 +Analogue 2	**+**	**- 649.87**
Hsp90 +Analogue 9	**+**	**- 650.64**

Table 8: Docking calculation between Hsp90 and mutant p53 after Hsp90 inhibition

The Table 8 summarized all the docking calculations and inferred the differences of docking energy before and after inhibition. Before inhibition the binding energy was -668.62 and after inhibition it has been changed accordingly with respect to the inhibitors docked. The energy value changed to less negative. As previously stated the more negative e-value the more the stable the complex would. Now according to that the stability of p53-Hsp90 binding complex became weaker upon inhibition. The reason may be due to the conformational changes of Hsp90 made by the inhibitor. When the inhibitor bind to the Hsp90 N-terminal domain, there must be some conformational changes to Hsp90 structure, because of which it had failed to form a stable complex with mutant p53.And this is what we needed, that means the inhibition not only interrupting catalytic activity but also inducing the conformational changes. Thus the objective set from the start of this work seems to be fulfilled computationally.

Chapter 5:
Conclusion
And
Future Perspectives

5.1 Conclusions

Protein-protein interactions and protein –ligand interactions play a crucial role in biological functions.Hsp90.The results stated that Hsp90 alone is not responsible for its chaperoning activity and malignancy but moreover it's a chaperoning complex with Hsp70 and Hsp40 that works simultaneously. Different domains of Hsp90 showed different affinity towards p53 (both wild type and mutant).Hsp90 showed more affinity towards wild type p53 as the docking calculations made by hex 6.3 i.e. -1170.76 kcal/mol, while in complex with Hsp70 and hsp40 expressed high affinity towards mutant p53 i.e. -834.01.Also the results of Ligplot analysis visualized the residues involves in hydrogen bonding, hydrophobic interaction and even the distance between the residues is found to be in angstrom unit. These finding confirmed the establishment of Hsp90 and its client p53 interaction and Involvement of the multi chaperoning complex in mutant p53 stability. Inhibition of Hsp90 may interrupt its chaperoning function as stated in all literatures. Here Geldanamycin is found to be a potent inhibitor among all the chosen ligands which scored high (-315.36 kcal/mol) on docking calculations made by Hex 6.3.As the literatures stated about its toxicity and poor solubility ,Geldanamycin was modified by Marvinsketch and formed 10 Analogues. Upon docking the analogues with Hsp90 by Hex, Analogue 2 scored high (-286.20 kcal/mol) but not better than original one. But upon docking by Autodock, Analogue 9 scored the best (-9.64kcal/mol) even better than original Geldanamycin. Other Analogue 5 (-9.60kcal/mol) and Analogue 1 (-9.63 kcal/mol) showed good results than original Geldanamycin (-9.53 kcal/mol).Also in terms of Ki (Inhibition constant) calculation all the three outnumbered Geldanamycin by exhibiting less concentration for inhibition. Ki is the concentration at which half of the maximum inhibition occurs. If the Ki is less that means less concentration can do the inhibition, and which means less cytotoxic and side effects of the ligand

molecules. Again the docking results of the inhibited Hsp90 complex with mutant p53 explicit the decrease in binding affinity between the Hsp90 and Mutant p53.It lead to make the assumption that may be the inhibitor upon inhibition changes the conformation of Hsp90 ,because of which the affinity goes down. There may be some changes in conformation so that the mutant p53 could not bind to Hsp90 with higher affinity. The above said results clearly stated that not only the inhibitor inhibited its catalytic activity but also interrupt its chaperoning action by interfering in the interaction between Hsp90 and Mutant p53.

5.2 Future perspectives

All the above findings are established with the help of promising, highly developed and reliable tools and software of computational biology. In this modern era of Insilco, every work is first tested by Virtual screening or Insilico designing, then only it goes for invitro and invivo analysis. The protocol follows like Insilico designing, Insitu designing, Invitro analysis and final invivo analysis. Likewise analogue 2 and Analogue 9 should undergo QSAR analysis and further the whole complex should be studied under molecular dynamic simulations. This would put the above findings in real dynamics to see the changes. And after simulation it can further go for invitro analysis.Hsp90 as a therapeutic target is not new but it shows new exciting results.

The crucial biological functions performed by HSP90 and the dependency of cancer cells on the marked functions of Hsp90 make itself as an attractive target for anti-cancer chemotherapeutics. Among the trademarks of cancer, up-regulation of growth signals and apoptotic interruption are the most vital. As maximum growth signals rely on Hsp90 for their functional stability, Hsp90 become an ideal molecule to interfere in complicated web of oncogenic pathways. Therefore, drugs targeting Hsp90 are more advantageous than the oncogene pathway inhibitors.

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